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Rab35 regulates neurite outgrowth and cell shape

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ABSTRACT

Recent studies have identified Rab35 in the endocytic pathway and as a regulator of cytokinesis; however its molecular mechanisms are currently unknown. Here, we find that Rab35 colocalizes with actin filaments and with Cdc42, Rac1 and RhoA, and that Rab35 can activate Cdc42 both in vivo and in vitro. We find activated Rab35 stimulates neurite outgrowth in PC12 and N1E-115 cells via a Cdc42-dependent pathway and that siRNA knockdown of Rab35 activity abolishes neurite outgrowth in these cell lines. We conclude that one function of Rab35 is to regulate Rho-family GTPases and that this role has consequences for neurite outgrowth.

Structured summary:
MINT-7012081: *Rac1*(uniprotkb:P63000) and *Rab 35* (uniprotkb:Q15286) *colocalize* (MI:0403) by *fluorescence microscopy* (MI:0416)
MINT-7012070: *actin* (uniprotkb:P60709) and *Rab 35* (uniprotkb:Q15286) *colocalize* (MI:0403) by *fluorescence microscopy* (MI:0416)
MINT-7012095: *cdc42* (uniprotkb:P60953) and *Rab 35* (uniprotkb:Q15286) *colocalize* (MI:0403) by *fluorescence microscopy* (MI:0416)

1. Introduction

Rab35 is found on the cell surface and endosomes, where it has been proposed to play a role in recycling of the transferrin and other receptors [1,2]. Intriguingly, there is also evidence supporting a role for Rab35 in cytokinesis [1]. Both cytokinesis [3] and movements of endosomes [4–6] depend on interactions of membranes with cytoskeletal elements, thus a role for Rab35 in the regulation of cytoskeletal organization would be an attractive hypothesis consistent with existing data. There is ample precedent for such interactions in membrane trafficking events. Rab7 can recruit the dynein microtubule motor to late endosomes [7], while Rab27 regulates melanosome motility via recruitment of myosin Va [8]. Rac1, RhoA and Cdc42 regulate changes in cell shape including neurite outgrowth and cytokinesis via modifications to actin cytoskeleton [9].

Nothing is known about the molecular mechanisms by which Rab35 may act. In this study, we pose and test the hypothesis that Rab35 is a regulator of actin cytoskeleton. We find that Rab35 can modulate cell shape, including an essential role in neurite outgrowth. We also find that Rab35 can activate Cdc42, and that dominant-negative Cdc42 can block Rab35-induced phenotypes, suggesting that at least part of the function of Rab35 is mediated via activation of Rho-family GTPases.

2. Materials and methods

2.1. Cells and reagents

Baby Hamster Kidney cells (BHK-21) and mouse neuroblastoma (N1E-115) cells were maintained as previously described [10,11]. Rat adrenal pheochromocytoma (PC12) cells were maintained in DMEM from Wisent (Rocklin, CA) with 10% horse serum, penicillin and streptavidin antibiotics, L-glutamine and incubated in a humidified incubator with 5% CO_2 at 37 °C.

Cells were transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions and analyzed 24 h after transfection unless stated otherwise. The 9E10 monoclonal antibody against myc was used. All remaining reagents were purchased from Sigma unless otherwise indicated.

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2.2. Plasmids

Creation of the GFP-Rab35 plasmid is described in [12]. Rab35 S22N and Rab35 Q67L were obtained by site-directed mutagenesis. Rab35 was cloned into a mRFP-C2 vector to obtain RFP-Rab35 and in a pRK5myc vector to obtain myc-Rab35. GST-CRIB, myc-Rac1-N17, myc-RhoA-N19 vectors are described in [13]. RhoA, Rac1, Cdc42 and Cdc42-N17 were subcloned into pEGFP vector (Clontech) from pRk5myc.

2.3. Differentiation assay

Prior to differentiation, N1E-115 cells were plated on coverslips coated with laminin and differentiation was induced by 36 h serum starvation [11]. PC12 cells were plated on coverslips coated with Poly-D-lysine (PDL) and differentiation was induced by the addition of 10 ng/ml of nerve growth factor (NGF) for 48 h [14]. Differentiation was induced immediately after transfection.

2.4. Image acquisition and analysis

Images of cells were taken on a confocal LSM 510 microscope from Carl Zeiss Inc. (Thornwood, NY). BHK cell shape was quantified by outlining the cells with LSM Image Browser software (Carl Zeiss) and measuring the ratio between perimeter and area. Neurite extension in PC12 and N1E-115 cells was quantified by calculating the fraction of cells containing neurites; cell extensions were considered as neurites when their length was equal or greater than the diameter of the cell body.

2.5. Cell silencing

N1E-115 cells were plated on coverslips coated with laminin in 24 well plates and transfected 20 h post-plating with 37.5 ng siRNA and 3 µl of HiPerFect according to the manufacturer's instructions (Qiagen, Düsseldorf, Germany). Thirty hours later, new medium without serum was added to initiate differentiation and cells were further incubated for 48 h.

2.6. Activation assay

Activation of Cdc42 was assayed with the CRIB domain of PAK using a protocol adapted from [13]. BHK cells lysates expressing myc-Cdc42 and the various mutants of GFP-Rab35 were obtained by lysing cells in 1% NP40, followed by centrifugation in an Eppendorf microcentrifuge at 15 min 13000 rpm to pellet the nuclei. Glutathione sepharose beads from GE-Healthcare (Piscataway, NJ) were coupled with purified GST-CRIB and washed. Beads were incubated with the lysates for 45 min at 4 °C. Cell lysates for positive control condition were meanwhile incubated 15 min at 30 °C in the presence of 100 µM GTPγS, followed by incubation for 30 min with GST-CRIB coupled beads at 4 °C. Beads were then washed and resuspended in sample buffer. Myc-Cdc42 binding to GST-CRIB was assessed by SDS–PAGE/Western blot and normalized to total myc-Cdc42 to account for transfection efficiency. Band density was quantified using Image J software (NIH).

3. Results

3.1. Colocalization of Rab35 with actin

By light microscopy, we found GFP-Rab35 throughout the cell with additional concentration in punctate structures (Fig. 1), consistent with previous studies which had localized GFP-Rab35 to endosomes and cell surface [1]. Some Rab35 staining was also found near cell rims and extensions reminiscent of the pattern of actin staining (Fig. 1A–F). We used a membrane fractionation assay [15] to test if there was a major additional cytosolic component of Rab35 not associated with membranes. Fifty-seven percent of Rab35 was membrane associated in BHK cells as assessed by high

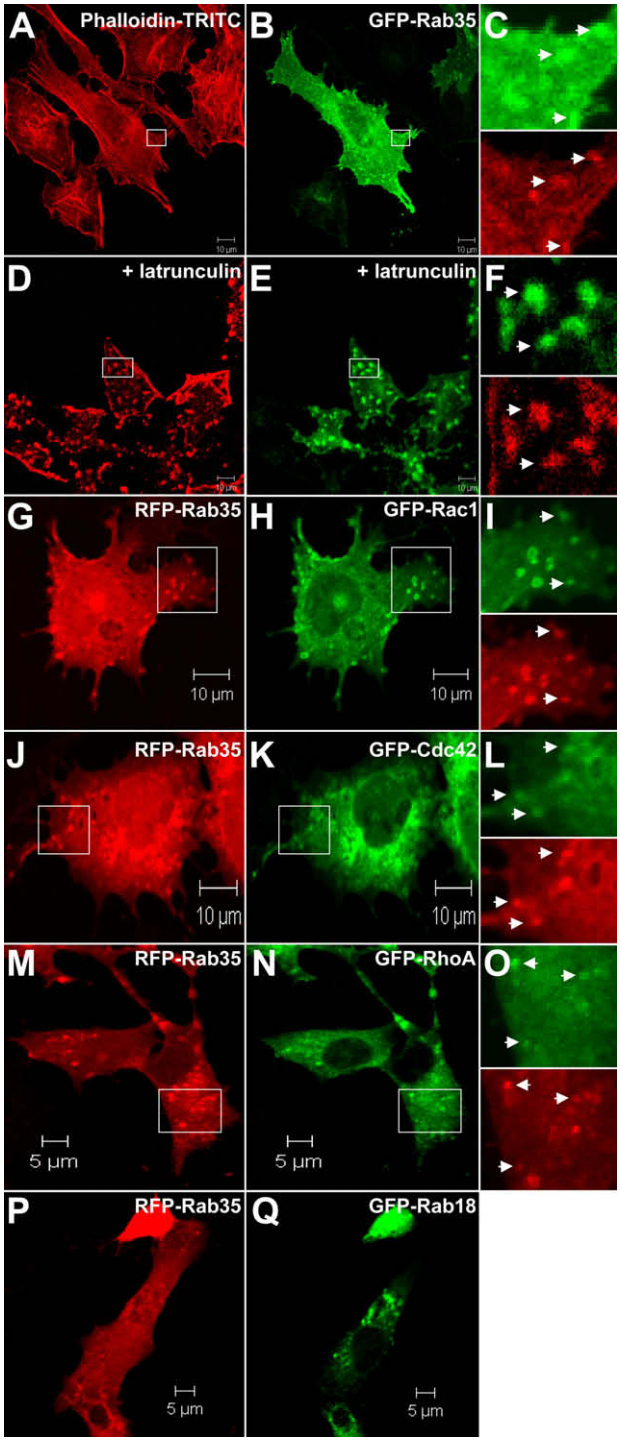

Fig. 1. Colocalization of Rab35 with actin and small GTPases. (A–F) BHK cells were transfected with GFP-Rab35, fixed at 36 h post-transfection, and stained with 50 µg/ml phalloidin-TRITC for 1 h. The inset shows a magnification of the cell, highlighting the colocalization between Rab35 and actin. (D–F) BHK cells were treated with 1 µM Latrunculin B for 1 h followed by phalloidin staining. Note that Rab35 continues to co-localize even after redistribution of actin. (G–Q) BHK cells were transfected with RFP-Rab35 and GFP-Rac1 (G–I), GFP-Cdc42 (J–L), GFP-RhoA (M–O) and GFP-Rab18 (P,Q) for 36 h prior to fixation and staining and magnification of their colocalization (I, L, O).

speed centrifugation. Similar results were obtained for Rab35Q67L (57%) whereas Rab35S22N was mostly cytosolic (89%) (data not shown).

We then tested whether a fraction of GFP-Rab35 colocalized with actin by staining transfected cells with phalloidin. We found colocalization at the edge of cells, on punctate structures throughout the cell and to a lesser extent on apparent stress fibers (see Fig. 1A and B). Treating cells with the actin-depolymerizing drug latrunculin B redistributed the actin into patches. Interestingly, Rab35 was strongly redistributed onto these patches (Fig. 1D–F). From these data we conclude that a substantial fraction of intracellular Rab35 is found near actin filaments.

3.2. Colocalization of Rab35 with Rho-family GTPases

RFP-Rab35 colocalized well with GFP-Rac1 and GFP-Cdc42 in BHK cells (Fig. 1G–L) on cell surface ruffles as well as internal structures. Colocalization on large vacuolar structures, potentially Rac1-induced macropinosomes, was frequently seen when RFP-Rab35 was co-expressed with GFP-Rac1, while smaller internal structures were seen when Rab35 was co-expressed with GFP-Cdc42. Interestingly, co-expression with RFP-Rab35 appeared to increase recruitment of both GFP-Cdc42 and GFP-Rac1 to internal membranes (data not shown). Colocalization with GFP-RhoA was less clear but could be detected in some BHK cells (Fig. 1M–O). RFP-Rab35 did not co-localize with a negative control (GFP-Rab18 [12]; Fig. 1P and Q). Similar colocalization of GFP-Rab35 with GFP-Rac1 and GFP-Cdc42 were obtained in HeLa cells (data not shown).

3.3. Rab35 induces changes in cell morphology

Striking alterations of cell shape took place in BHK cells in which GFP-Rab35 was strongly expressed. These flat cells have a typically fibroblast-like morphology (see mock-transfected cells Fig. 2A) [16]. Surprisingly, upon expression of Rab35Q67L or strong overexpression of GFP-Rab35, BHK cells produced unusual long cell extensions (Fig. 2B and D). Those cell extensions were few in number and spanned a greater distance than normally covered by BHK cells, thus they did not result from cell shrinkage which typically produces many slender but short cell extensions [17]. We observed as well that overexpressed Rab35S22N reduced the number of cell extensions (Fig. 2C). As a quantitative measure of cell shape, we measured the ratio of perimeter to area for 100 cells in each condition. This ratio is higher in cells with long and thin extensions. As observed (Fig. 2E), wild-type- and Rab35Q67L overexpression in BHK induced a statistically significant ($P < 0.001$) increase in this ratio, of 2- and 2.5-fold compare to the mock-transfected condition. Transfection with Rab35S22N produced a small measured reduction which was not statistically significant (Fig. 2E).

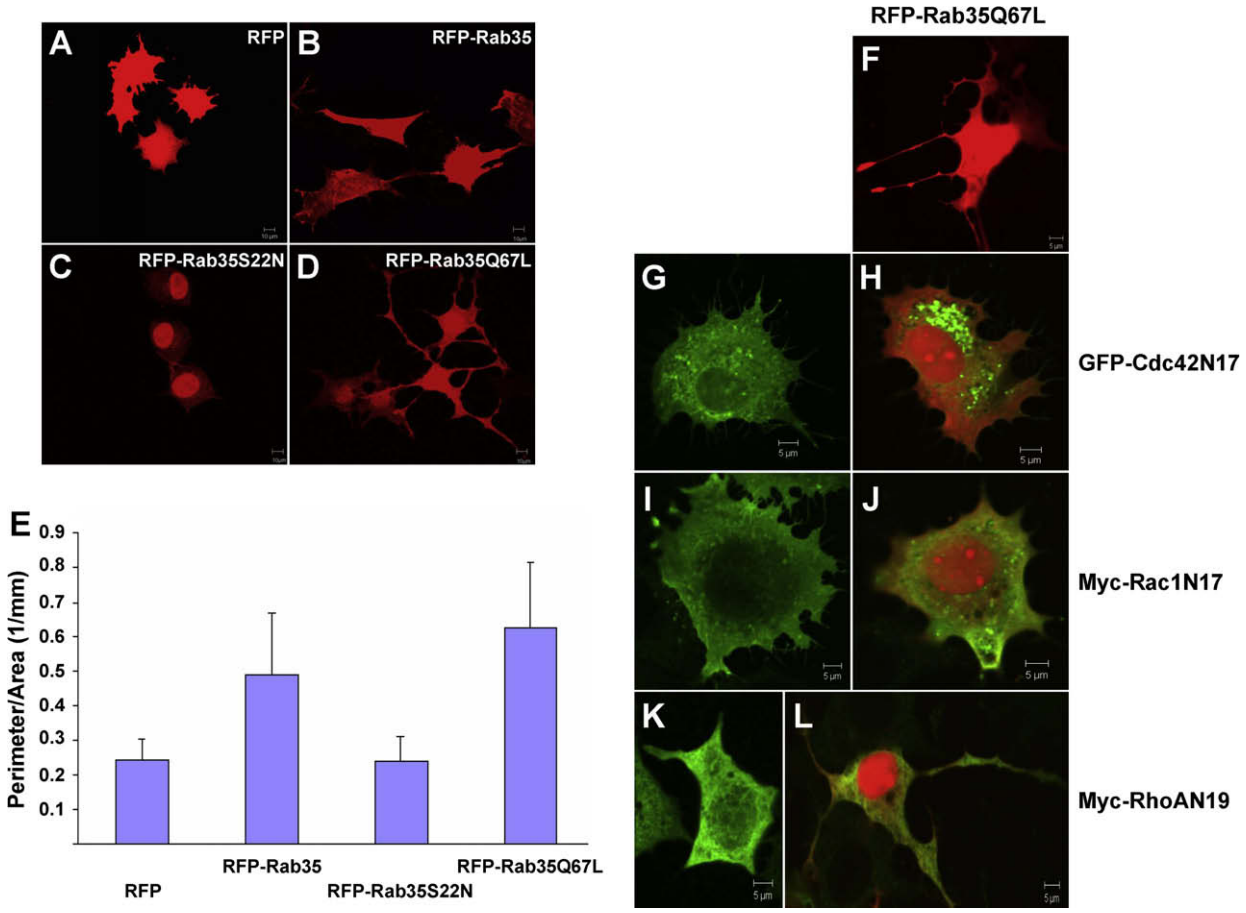

Fig. 2. Effect of Rab35 and small GTPases on BHK morphology. (A–D) BHK cells were transfected as indicated with RFP, RFP-Rab35, RFP-Rab35S22N or RFP-Rab35Q67L and cells fixed 36 h post-transfection. (E) Perimeter/area was measured for 100 cells for each of the conditions in (A–D). Increases of this ratio for RFP-Rab35 and RFP-Rab35Q67L relative to RFP vector were statistically significant by *t*-test ($P < 0.0001$), whereas RFP-Rab35S22N was not ($P = 0.66$). (F) BHK cells transfected with RFP-Rab35Q67L. (G and H) BHK cells transfected with GFP-Cdc42N17 and (H) co-transfected with GFP-Cdc42N17 and RFP-Rab35Q67L, no neurite extension was observed. (I, J) BHK cells transfected with myc-Rac1N17 and (J) co-transfected with myc-Rac1N17 and RFP-Rab35Q67L, no neurite extension was observed. (K, L) BHK cells transfected with myc-RhoAN19 and (L) co-transfected with myc-RhoAN19 and RFP-Rab35Q67L. In these cells myc-RhoAN19 was unable to prevent neurite extension induced by RFP-Rab35Q67L.

3.4. Involvement of Rac1 and Cdc42 in Rab35-induced cell extensions

It is well known that some Rho-family GTPases are implicated in cell cytoskeleton remodeling [18]. Thus, we investigated whether there was an involvement of Rho-family GTPases in Rab35-mediated cell extensions. We co-transfected BHK cells with Rab35Q67L and dominant-negative forms of Cdc42, Rac1 and RhoA. Interestingly, we found that both Cdc42N17 and Rac1N17 prevented the formation of cell extensions (Fig. 2F–J), while RhoAN19 had no visible effect on the morphology (Fig. 2K and L). This was confirmed upon quantification (200–400 cells each condition). Whereas 84.8% of cells expressing RFP-Rab35Q67L possessed extensions, this was reduced to 3.9% of cells in which GFP-Cdc42N17 was co-expressed, or 5.9% of cells in which Myc-Rac1N17 was co-expressed. Coexpression of Myc-RhoAN19 had little if any, effect (83.1% cells with extensions). To determine if Rab35 can regulate Rho-family GTPases we tested for activation in vitro of Cdc42 and Rac1. Fig. 3A shows that upon expression of Rab35 or Rab35Q67L, Cdc42 activation is increased 3-fold over control conditions (Fig. 3B). Surprisingly, 2-fold activation was induced with Rab35S22N. No significant activation was found with Rac1 (data not shown).

3.5. Rab35 and neurite outgrowth

We investigated the possibility that Rab35 might be involved in the formation of physiological extensions such as neurites [19]. N1E-115 neuroblastoma cells were transfected with Rab35 wild-type or mutant and control constructs and neurite outgrowth scored after 36 h as described in Section 2 (Fig. 4A–D). We observed that transfection of Rab35Q67L increased the fraction of cells with neurites by more than 3-fold compare to control, while neurite outgrowth was inhibited in cells transfected with Rab35S22N (Fig. 4E). Similar results were found in cells transfected with Rab35 wild-type or mutants and scored after 48 h of differentiation (Supplementary Fig. S1) and in PC12 cell (Supplementary Fig. S2). Rab35 induced neurite outgrowth was dependant on Rac1 and Cdc42 but not RhoA in N1E-115 cells (Supplementary Fig. S3) and PC12 cells (data not shown). We then examined the effect of reduction of endogenous Rab35 expression with siRNAs (see Section 2). Differentiation was induced 30 h after transfection with siRNAs, and neurite outgrowth was analyzed at 78 h post-transfection (Fig. 4F–H). The quantification clearly shows that down-regulation of Rab35 in N1E-115 significantly reduces post-differentiation neurite outgrowth (Fig. 4I).

4. Discussion

Roles for Rab35 have been proposed in endocytosis and in cytokinesis, suggesting the possibility that Rab35 functions to regulate cytoskeleton, which plays a key role in both processes. In this study, we have determined: (1) that intracellular Rab35 is found in proximity to actin, and that the Rab35 distribution changes when the actin cytoskeleton is disrupted, (2) that overexpression or inhibition of Rab35 activity can alter cell shape, consistent with functional interactions with actin cytoskeleton, (3) that Rab35 colocalizes with Rho-family GTPases, (4) that Rab35 activates Cdc42, and (5) that Rab35 is a positive regulator of neurite outgrowth. These findings suggest that Rab35 regulates remodeling of actin cytoskeleton, at least in part via regulation of Rho-family

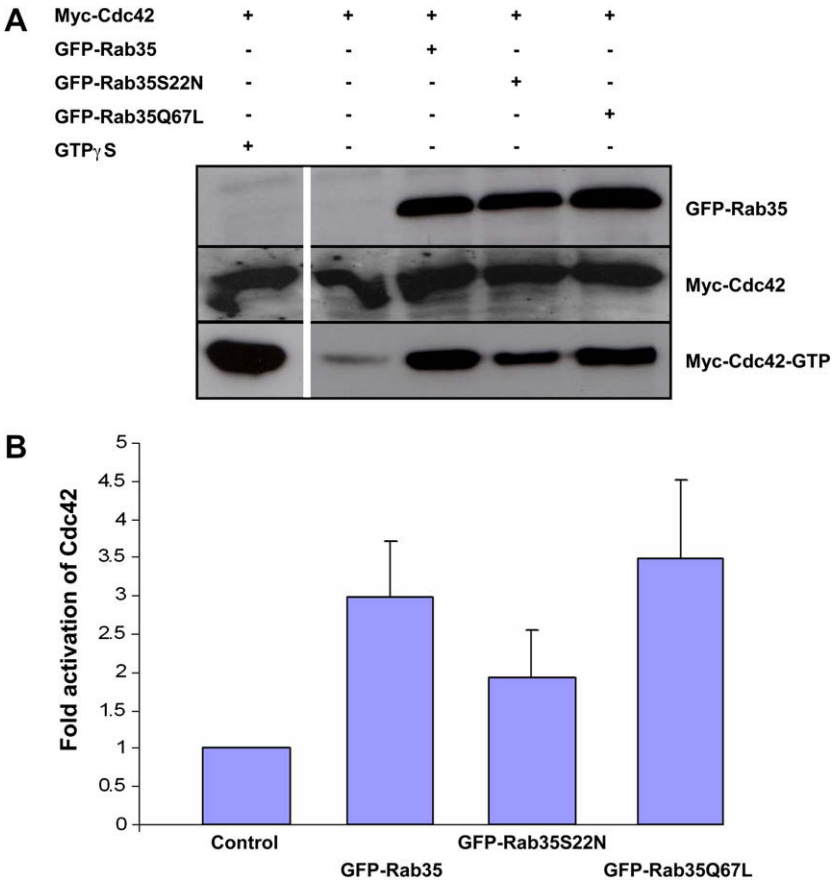

Fig. 3. Cdc42 activation assay in BHK cells expressing various Rab35 constructs. (A) Western blot analysis of GFP-Rab35 expression (top; αGFP), Cdc42 in lysate (middle; αmyc) and Cdc42-GTP bound to a GST-CRIB column (bottom; αmyc). (B) Quantitation of GTP bound myc-Cdc42/total Cdc42 as described in Section 2 normalized to serum-starved control, $n = 5$.

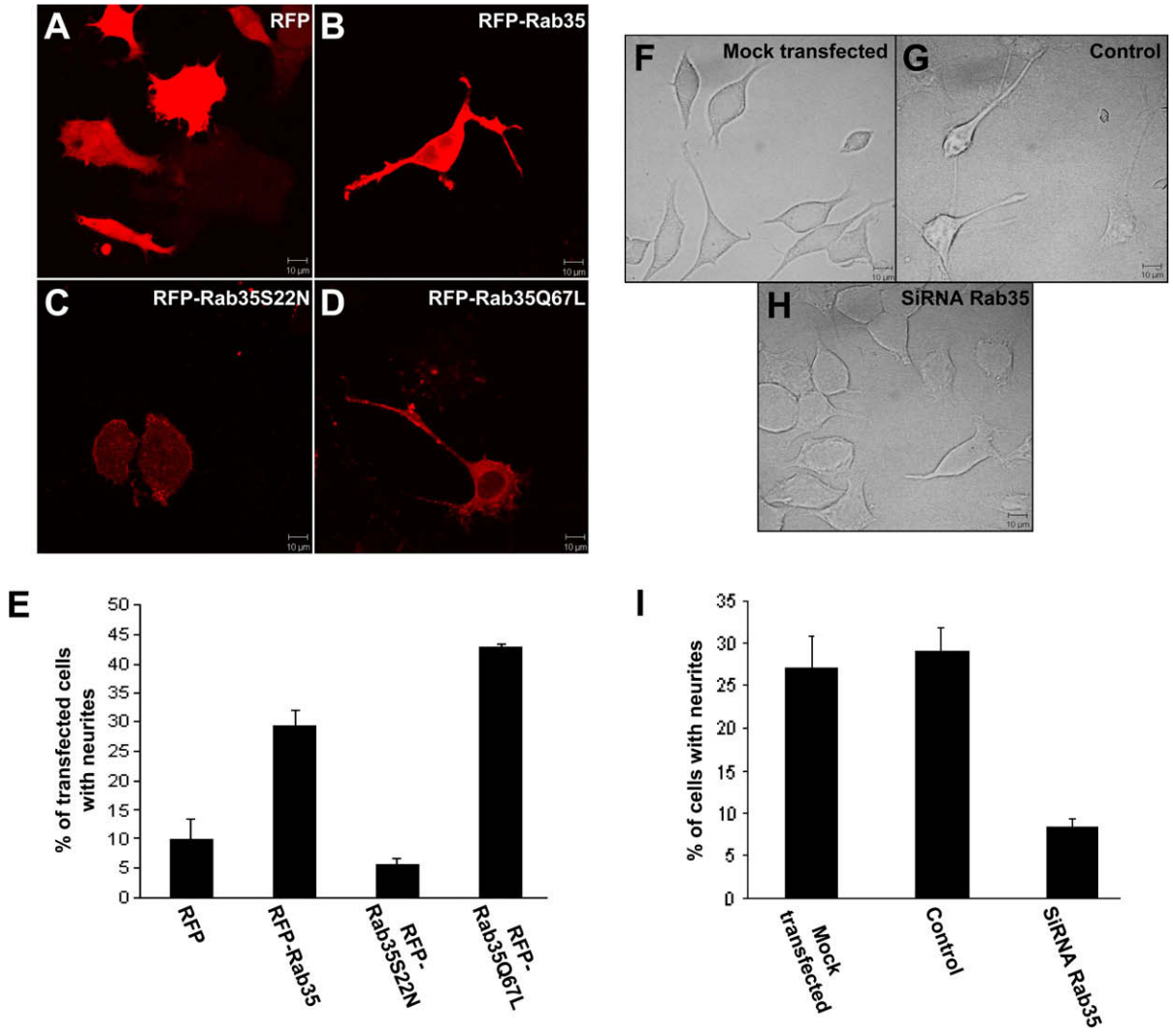

Fig. 4. Effect of Rab35 overexpression and silencing on neurite outgrowth in N1E-115 cells. (A–D) N1E-115 cells were transfected with RFP, RFP-Rab35, RFP-Rab35Q67L or RFP-Rab35S22N (as indicated). Cells were fixed 36 h post-transfection and images acquired. (E) Neurite outgrowth was quantitated in 60 cells/condition as described in Section 2. (F) Phase contrast pictures of differentiated cells (48 h post-differentiation), mock-transfected with siRNA, without silencing (G) or transfected with siRNA directed against Rab35 72 h prior to fixation (H). (I) Quantification as described in Section 2 of neurite outgrowth in 100 cells/condition in each of three independent experiments.

GTPases. Rab35 may be implicated in both cytokinesis, which involves remodelling of actin and tubulin cytoskeleton, and in endocytosis, which can involve interactions both with actin and with Rho-family GTPases.

We find not only that exogenous expression of Rab35 can influence cell shape and neurite outgrowth but also that down-regulation of endogenous Rab35 synthesis inhibits neurite outgrowth. This strongly supports that Rab35 plays a physiological role in the formation of some membrane process including neurites, with this role mediated in part via Rac1 and Cdc42. None of the previous studies have investigated potential roles for Rab35 in regulating cell shape or neurite outgrowth. Regulation of Rho-family GTPases by Rab35 suggests the hypothesis that there are conserved mechanisms of action in some of Rab35's role in the seemingly diverse processes of cell division, neurite outgrowth and endocytosis, all of which are processes influenced by Rho-family GTPases.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2009.03.012.

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